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Developmental Basis of Phenotypic Variation in Egg Production in a Colonial Ascidian: Primary Oocyte Production *Versus* Oocyte Development

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Abstract. Colonies of the ascidian *Botryllus schlosseri* (a cyclical hermaphrodite) exhibit extreme variability in egg production, and there is a large genetic component to this phenotypic variation. Therefore, the developmental bases of variation among different genotypes was investigated. Colonies differing in egg production (assayed as number of eggs per asexual bud) were cultured in a common garden experiment, and buds were collected and fixed early in the reproductive cycle. The buds were serially sectioned, and the number and size of the oocytes in the developing ovaries were determined for the different genotypes. Because the buds were collected prior to the onset of vitellogenesis, they contained oocytes at the three previtellogenic stages. In reproductive colonies (>0.7 eggs per bud), there were negative relationships between the final number of eggs per bud and (1) the total number of oocytes present, (2) the number of stage 1 oocytes present, and (3) the number of stage 2 oocytes present. There was no relationship between these parameters in nonreproductive colonies (<0.3 eggs per bud). In contrast, the number of stage 3 oocytes per bud was positively correlated with the final number of eggs per bud in both reproductive and nonreproductive colonies. In reproductive animals there was a negative relationship between the total number of oocytes per bud and the percentage of oocytes at stage 3 in oogenesis. A principal component analysis revealed that a single vector equally weighted for the number of eggs per bud, the total number of oocytes per bud, and the percentage of oocytes at stage 3 accounted for 84% of the observed variation in reproductive colonies. These data indicate that the phenotypic variation in egg production among the *B. schlosseri* colonies in the Damariscotta River, Maine, is controlled by genetic variation in both the number of oocytes that populate developing ovaries, and the percentage of oocytes that reach stage 3 in oogenesis.

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Introduction

The production of ova is an exceedingly important aspect of the life-history strategy of any female or hermaphroditic organism. Because egg production is a primary determinant of evolutionary fitness, the mechanisms by which genetic and environmental factors may produce variation in fecundity among individuals must be assessed. Nutritional studies in teleosts and lizards have demonstrated that suboptimal diet reduces the number of ovulated eggs, either by reducing the number of oocytes that enter vitellogenesis, or by increasing oocyte atresia (Mendez-de la Cruz *et al.*, 1993; Tyler and Sumpter, 1996). Although a genetic basis for the variation in female fecundity has been demonstrated in many taxa, analysis of the genetic and developmental mechanisms that produce phenotypic variation in egg production has largely lagged behind (but see Land and Robinson, 1985, for studies in sheep). The genetic and developmental mechanisms controlling egg production in marine invertebrates are unknown and unstudied.

The colonial marine ascidian *Botryllus schlosseri* has characteristics that make it a desirable candidate for an investigation of the developmental mechanisms controlling intraspecific variation in egg production. First, both ovarian development and oocyte maturation occur in a number of repeated cycles, once an animal attains sexual maturity (Milkman, 1967; Sabbadin and Zaniolo, 1979; Manni *et al.*,

1994; Yund *et al.*, 1997). Colonies grow by asexual budding, and each developing bud within the colony has the capacity to form a pair of ovaries and testes (though the oogenic potential of buds varies within a colony; Sabbadin and Zaniolo, 1979). Consequently, the number of eggs produced per bud, rather than total colony-wide egg production, is generally used to assay relative female fecundity. Secondly, phenotypic variation in egg production occurs both within and among populations. The population in the Damariscotta River, Maine, exhibits a continuous range of variation, from 0 to 6 eggs per bud (Yund *et al.*, 1997). In contrast, the population in the Eel Pond at Woods Hole, Massachusetts, has a bimodal distribution in egg production, with the low mode at 2 eggs per bud and the high mode at 10 eggs per bud (Grosberg, 1988). Finally, in both populations, phenotypic variation in egg production is known to have both genetic and environmental components (Grosberg, 1988; Yund *et al.*, 1997).

In this report, we examine both ovarian development and oocyte maturation in the population of *B. schlosseri* living in the Damariscotta River. We evaluate three mutually compatible hypotheses for the production of a continuous range of phenotypes in the number of eggs produced per bud: (1) the number of oocytes that populate the developing ovaries within a bud varies by genotype, and a fixed percentage of these oocytes mature; (2) a fixed number of oocytes populate a developing bud, but different percentages of the oocytes mature in different genotypes; (3) a fixed number of oocytes populate a developing bud, and a fixed percentage of them mature, but the number of maturing oocytes that become atretic varies by genotype.

Materials and Methods

Study organism

Colonies of *Botryllus schlosseri* are composed of asexually produced zooids arranged in clusters, or systems, with all zooids in a system sharing a common exhalant siphon. Throughout the life of a colony, all of the zooids undergo a series of synchronous, overlapping sexual and asexual cycles. The asexual zooid replacement cycle starts when a new generation of zooids, called buds, form on the side of existing zooids (Berrill, 1941). After about 14 days of development and growth at 16°C, these buds swell and their inhalant siphons open. As the new zooids take over the function of the previous generation of zooids, which are quickly resorbed, they enter into their sexual cycle. The reproductive cycle includes the internal fertilization of the mature eggs soon after the inhalant siphons open (Milkman, 1967); the continuous release of sperm starting 16 h later (Stewart-Savage and Yund, 1997); and the brooding of the developing embryos, which are released just before the zooids degenerate (Milkman, 1967). *B. schlosseri* colonies can be staged according to their 14-day bud development cycle (Berrill, 1941; Izzard, 1973), or according to their 7-day reproductive cycle (Milkman, 1967). Because the next generation of buds is formed halfway through the bud development cycle, there are always three generations of zooids in a colony. During most of the reproductive cycle these three generations are adult zooid, primary bud, and a younger secondary bud. When the zooids of a new generation open their siphons (stage 0 according to Milkman, 1967), the colony contains degenerating zooids, newly opened zooids (which were primary buds), and primary buds (which were secondary buds).

Colony staging and bud collection

The *B. schlosseri* colonies used in this study were collected from the Damariscotta River, Maine. Animals were grown on glass microscope slides in the flowing seawater system at the University of Maine's Darling Marine Center in a common garden experiment, and thus experienced identical environmental conditions. Colonies were staged according to Milkman's (1967) six-stage reproductive cycle: colonies are at stage 0–1 during the first 24 h after a new generation of zooids open their siphons and at stage 3–4 when the expanding primary buds contain oocytes at the end of vitellogenesis. Over the experimental period, colonies at stage 3–4 were assayed for the number of zooids, the number of primary buds per zooid, and the average number of mature eggs per bud. To assay overall genotypic egg production, the average number of mature eggs per bud was determined by recording the number of eggs per bud in 10 randomly chosen primary buds (Yund *et al.*, 1997). This subsampling technique should minimize the effect of intracolony variation in oogenic potential (Sabbadin and Zaniolo, 1979). When the colonies reached stage 0–1, the area containing the primary buds was collected by making midline cuts through two adjacent zooids and removing the entire tunic between them. For each colony, 10 pieces of tunic from at least two areas of the colony were collected and fixed (see below). The colonies were reassayed when they reached stage 3–4.

The excised buds were fixed in 2% glutaraldehyde in 20 m*M* TRIS-buffered seawater, pH 8.0. After being fixed for at least 24 h, the buds were rinsed in seawater and then in distilled water, and then stained with Harris hematoxylin (Sigma, St. Louis, MO) for 15 min. After a distilled water rinse, the buds were dehydrated and embedded in methacrylate plastic (JB-4, Polysciences, Warrington, PA). Blocks, which contained 3–5 buds from a colony, were serially sectioned at 6 μm. A calibrated reticle at 160× magnification was used to measure the largest diameter of the oocytes within a bud; the size of elliptical oocytes was determined by averaging the two dimensions. For each animal, the total number of oocytes and the number of oocytes at each stage

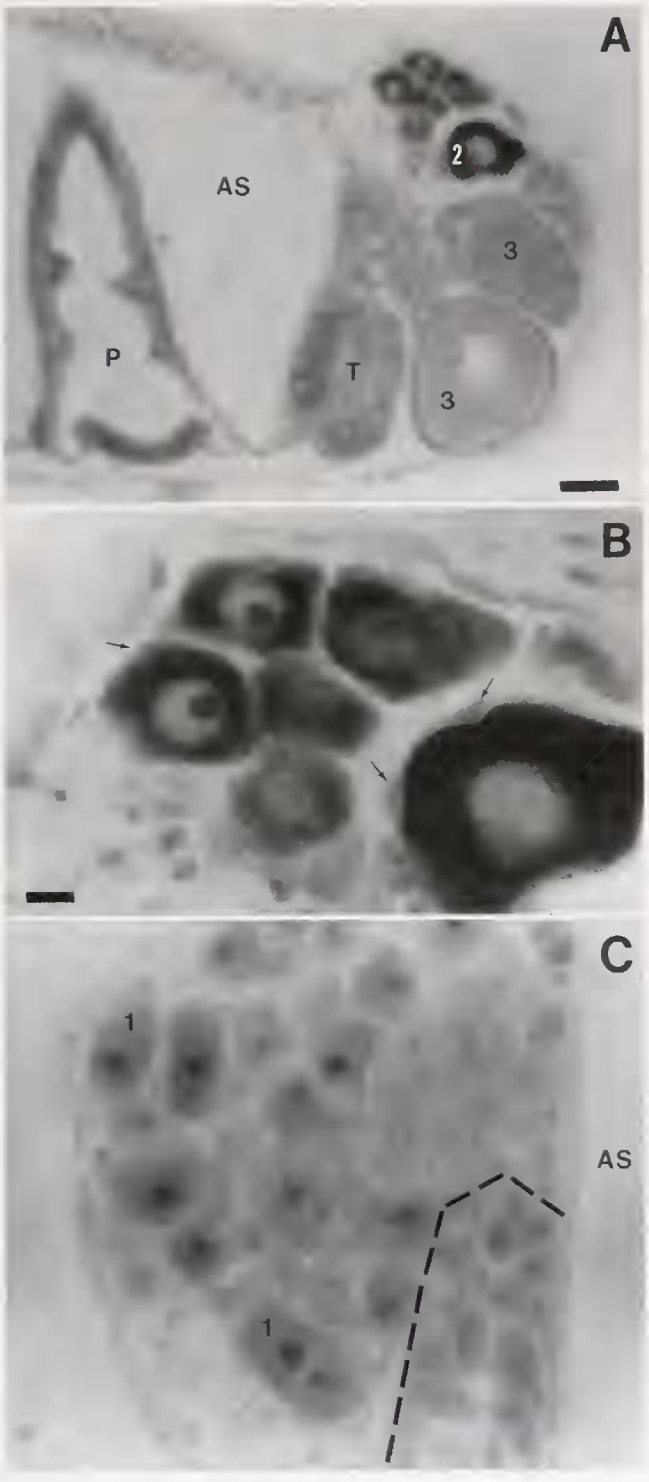

Figure 1. Micrographs of the different stages of oocyte development seen in developing buds of *Botryllus schlosseri* (stage 7 according to Izzard, 1973). See Results for details of oocyte staging. 1–3, stage 1–3 oocytes; AS, antral sac; P, developing pharynx; T, testis rudiment. (A) Cross section of a bud with a developing testes and stage 2 and 3 oocytes. The lightly stained stage 3 oocytes are surrounded by a darker, cuboidal follicular layer. 165×, bar = 50 μm. (B) Higher magnification of stage 2 oocytes shown in A. Small primary follicle cells (arrows) can be seen around each of the six stage 2 oocytes. 650×, bar = 10 μm. (C) Cross section of a bud containing only stage 1 oocytes. Notice the difference in cell size and nucleolar morphology between the stage 1 oocytes and the cells in the developing testes (area within broken line). 650×.

of oogenesis were determined by averaging the data from 6.4 ± 1.9 (X ± SD; range: 4–11) buds.

Because of the tradeoff between sexual reproduction and asexual growth (Yund *et al.*, 1997), the 20 colonies used in this study were all of medium size (110 ± 44 zooids, X ± SD) and had equal rates of asexual growth (1.6 ± 0.2 buds per zooid, X ± SD). To limit the effect of food supply on the number of eggs per bud (Grosberg, 1988; Yund *et al.*, 1997), all samples were collected over an 11-day period (27 June–8 July 1995). Six of the 20 colonies were producing few, if any, eggs. Because colony age and the environment can both affect egg production (Grosberg, 1988; Yund *et al.*, 1997) and we do not know if the lack of egg production in these colonies is the result of genetic factors, environmental factors, or a combination of both, we separated the colonies into "reproductive" (>0.7 eggs per bud) and "nonreproductive" (<0.3 eggs per bud) groups.

Results

Oocyte morphology and staging

The primary buds collected from *B. schlosseri* colonies between stages 0 and 1 in the reproductive cycle described by Milkman (1967) were, based on their histological appearance, at stage 7 according to Izzard's classification (1973). As expected, the ovaries of these developing buds contained only previtellogenic oocytes at three stages (Manni *et al.*, 1994) of oogenesis (Fig. 1). The small stage 1 oocytes (Fig. 1C) can be easily distinguished from the other cells in the developing ovary and from the testes rudiment by their larger size (10–15 μm *versus* 6–8 μm, respectively) and their large and prominent nucleolus. Stage 2 oocytes are characterized by an increase in both cell and nuclear size, and by an increase in the basophilia of the cytoplasm (Fig. 1A, B). Although Manni *et al.* (1994) described stage 2 oocytes as being 40–60 μm, we found definitive stage 2 oocytes that were only 20 μm. Stage 3 oocytes are distinguished from stage 2 oocytes by a further increase in oocyte and nuclear size, by a decrease in cytoplasmic basophilia, and by the presence of a cuboidal follicular layer (Fig. 1A). On the basis of these morphological criteria, stage 3 oocytes ranged in size from 60 to 100 μm. In the 126 buds sectioned, we observed only four atretic stage 3 oocytes (not shown). These oocytes were classified as atretic because test cells had migrated into oocyte cytoplasm, and the oocyte had no germinal vesicle. These four stage 3 oocytes were excluded from the data set.

Relationship between oocyte number and stage, and final egg number

As discussed in the Introduction, the average number of eggs produced in each bud (the colony's eggs-per-bud phenotype) may be controlled by the total number of oocytes within each developing bud. In reproductive colonies (>0.7 eggs per bud), the final eggs-per-bud phenotype is negatively related to the total number of oocytes within each bud, whereas in nonreproductive colonies (<0.3 eggs per bud) there is no relationship (Fig. 2A). This relationship indicates that the final number of eggs produced within each bud may be negatively regulated by the number of oocytes that populate the bud, but that the final determination of a colony's eggs-per-bud phenotype must occur during oogenesis.

To determine the stage or stages of oogenesis at which the final eggs-per-bud phenotype is determined, we examined the relationship between the number of oocytes at each stage of oogenesis and the final eggs-per-bud phenotype. In reproductive colonies, there is a negative relationship between the final eggs-per-bud phenotype and both the number of stage 1 and stage 2 oocytes per bud (Fig. 2B and C). In nonreproductive colonies, there is no relationship between the final eggs-per-bud phenotype and the number of stage 1 and 2 oocytes. The number of stage 3 oocytes per bud is positively correlated with the final eggs-per-bud phenotype in both reproductive and nonreproductive colonies (Fig. 2D).

The correlation of a colony's final eggs-per-bud phenotype with both the total number of oocytes in each bud (Fig. 2A) and the number of oocytes at stage 3 in oogenesis (Fig. 2D) indicates that the processes determining these two conditions may be coordinated. To determine the relationship between these processes, we converted the number of stage 3 oocytes per bud to a percentage to remove the negative relationship between the final eggs-per-bud phenotype and the total number of oocytes per bud. The number and percentage of oocytes in a bud at stage 3 in oogenesis are equivalent measures of oocyte maturation ($R = 0.797$). As seen in Figure 3, there is a negative relationship in reproductive colonies between the total number of oocytes per bud and the percentage of those oocytes that have reached stage 3 in oogenesis, whereas there is no relationship in nonreproductive colonies. When both variables are plotted against the final eggs-per-bud phenotype, the data points fall in the vicinity of a line (data not shown). A

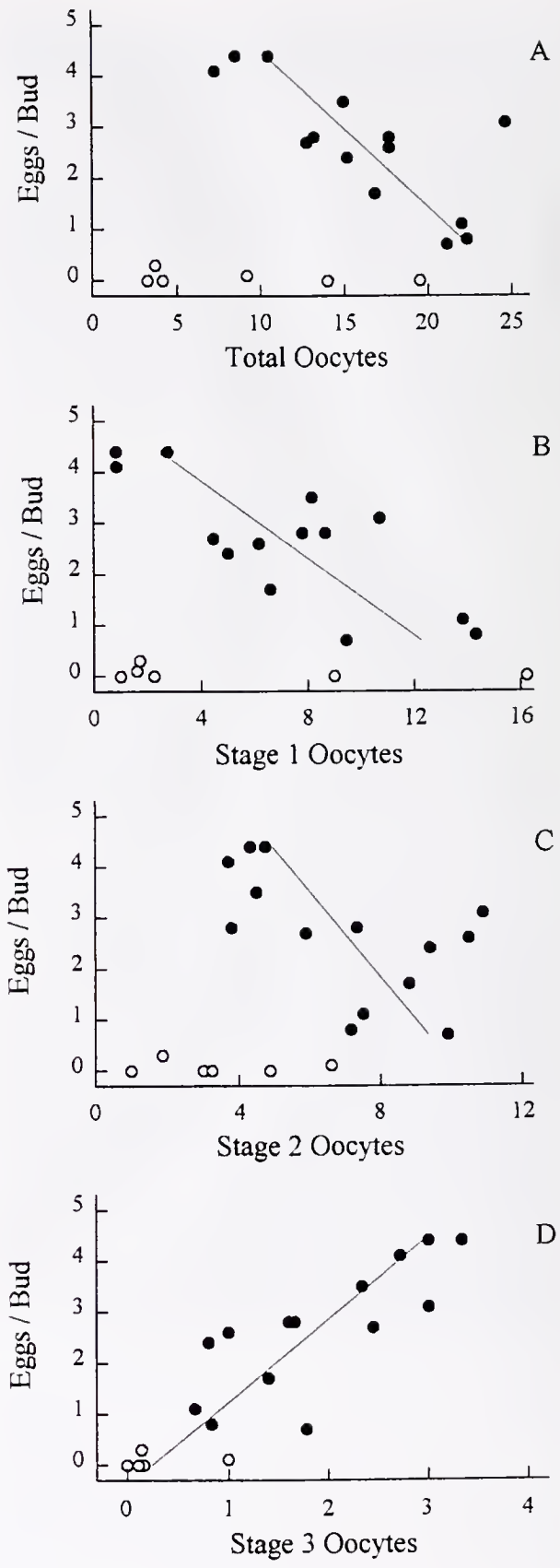

Figure 2. Relationship between the eggs-per-bud phenotype at Milkman stage 3 and the number of oocytes per bud at Milkman stage 0. See Results for details of oocyte staging. ○, nonreproductive colonies (<0.3 eggs per bud); ●, reproductive colonies (>0.7 eggs per bud). Lines are least squares linear regression of reproductive colonies in A–C and all colonies in D; A: $R = 0.756$, B: $R = 0.774$, C: $R = 0.568$, D: $R = 0.780$.

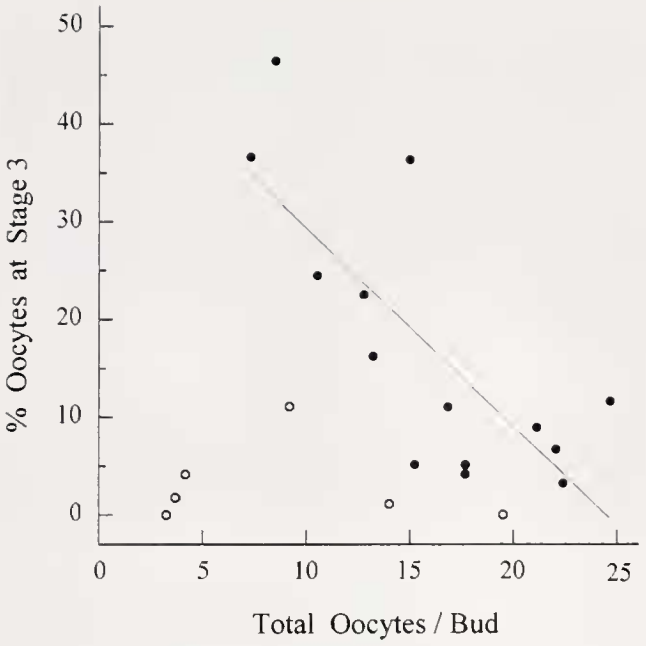

Figure 3. Relationship, at Milkman stage 0, between the total number of oocytes per bud and the percentage of oocytes at stage 3 in oogenesis. Line is least squares linear regression of reproductive colonies ($R = 0.763$). ○, nonreproductive colonies (<0.3 eggs per bud); ●, reproductive colonies (>0.7 eggs per bud).

principal component analysis (Table I) reveals that a single vector equally weighted for the three variables accounts for 84% of the variation.

Discussion

The strong relationship between a *B. schlosseri* colony's final eggs-per-bud phenotype and both the total number of oocytes that are in the developing ovary and the number of oocytes at stage 3 of oogenesis indicates that two separate mechanisms operate to determine the final number of eggs that a colony produces. In reproductive colonies, a variable number of oocytes populate a developing ovary, a variable percentage of those oocytes reach stage 3 in oogenesis, and the final eggs-per-bud phenotype is determined by the negative relationship between the two. Although the strong relationship between these two variables in reproductive colonies suggests that they may be genetically linked, the data from nonreproductive colonies demonstrate that the control of oogenesis can be uncoupled from the number of oocytes that populate a developing ovary.

Control of the number of oocytes within a developing bud

The development of the ovaries in *B. schlosseri* is not a one-time event, but occurs during each asexual cycle. Studies on the development of buds in *Botryllus* have demonstrated that germ cells are not seen in the developing bud until after it is vascularized (Izzard, 1973; Mukai and Watanabe, 1976; Sabbadin and Zaniolo, 1979; Manni *et al.*, 1995). Histological and genetic data indicate that germ cells do not arise from the bud epithelium but enter the developing bud from the blood. First, cells similar in morphology to stage 1 and stage 2 oocytes have been seen in the peripheral circulation during the time when germ cells start appearing in the developing buds (Izzard, 1968; Mukai and Watanabe, 1976; Sabbadin and Zaniolo, 1979; pers. obs.). Second, the migration of germ cells *via* the vascular system has been demonstrated by genetic assays. When two colonies that were genetically different for either pigment genes (Sabbadin and Zaniolo, 1979) or microsatellites (Pancer *et al.*, 1995) were allowed to become vascularly fused and were then separated, about 50% of the separated colonies produced gonads or offspring containing the same genetic component as the previously fused partner. It may be that the number of migrating germ cells that take up residence within a developing bud is genetically controlled.

In addition to the possibility that germ cell migration is genetically controlled, *B. schlosseri*'s reproductive life-history parameters indicate that mitosis of the germ cells must occur sometime during each bud cycle. As we have shown in this report, colonies that produce about 4 eggs per bud have only about 5–8 oocytes at stage 1 or 2 in each bud. Since *B. schlosseri* colonies produce about the same number of eggs per bud in each cycle (Yund *et al.*, 1997) and reproduce for up to 10 cycles (Grosberg, 1988), mitosis must be occurring within the germ cell population. If the total number of germ cells in a colony were fixed, either egg production would decline with age or the number of reproductive cycles would be low. The above observations indicate that the genetic mechanism that determines the total number of oocytes in each bud may operate by controlling the migration of germ cells into the developing buds, the number of mitotic divisions in the germ cells during each bud cycle, or both.

Table I

Principal component analysis of the three-way relationship between eggs-per-bud phenotype, total number of oocytes per bud, and percentage of oocytes at stage 3 in oogenesis

	Principal Components		
	PC1	PC2	PC3
Eigenvalue	2.52	0.24	0.24
Percent of variance	84.02	8.14	7.84
Cumulative percent of variance	84.02	92.16	100.00
Eigenvector loadings			
No. Eggs per bud	0.58	0.80	−0.18
No. Oocytes per bud	−0.58	0.56	0.60
% Oocytes at oogenesis stage 3	0.58	−0.24	0.78

Control of oogenesis

Oogenesis in *B. schlosseri* has been divided into five stages by Manni *et al.* (1994), with stages 1–3 being previtellogenic and stages 4–5 being vitellogenic. The nearly 1:1 relationship between the final eggs-per-bud phenotype and the number of stage 3 oocytes (Fig. 2D) indicates that all oocytes that reach stage 3 will continue on to stage 4 and undergo vitellogenesis. This 1:1 relationship also indicates that oocyte atresia after the initiation of vitellogenesis is not a genetic mechanism used to regulate oocyte number in *B. schlosseri*. The number of oocytes that complete oogenesis in *B. schlosseri* is apparently regulated at the transition between stages 2 and 3 of oogenesis, because the ovaries of nonreproductive colonies contain variable numbers of stage 1 and stage 2 oocytes, but few stage 3 oocytes (Fig. 2). Thus, germ cell migration and the early stages of oogenesis are occurring in these nonreproductive colonies, but the oocytes are prevented from entering stage 3 of oogenesis.

Modes of genetic inheritance of fecundity

The genetic control of the number of eggs per bud in *B. schlosseri* colonies shares striking similarities with the genetic control of the number of eggs ovulated by sheep. Populations of both animals exhibit a continuous range of phenotypes, and this phenotypic variation has a large genetic component. In the sheep, increased fecundity is a polygenetic trait in some breeds and the result of a single gene mutation in others. In the two sheep breeds where litter size is polygenetically controlled, the heritability of increased fecundity is about 0.50 (Hanrahan and Quirke, 1985; Mavrogenis, 1985). The heritability of litter size in sheep breeds not selected for increased fecundity has been estimated to be around 0.07 (Bradford, 1985). The heritability of these polygenetically based increases in sheep fecundity is similar to the broad-sense heritability calculated for the eggs-per-bud phenotypes of *B. schlosseri* in the Damariscotta River, Maine (Yund *et al.*, 1997).

Of the two populations of *B. schlosseri* in which the genetic basis of the eggs-per-bud phenotypes has been examined, only the population in Eel Pond at Woods Hole, Massachusetts, has both iteroparous (1–5 eggs per bud, reproduce multiple times) and semelparous (9–14 eggs per bud, die at end of first reproductive cycle) life-history morphs (Grosberg, 1988; pers. obs.). The inheritance of the two life-history morphs may be controlled by a single gene because the number of eggs produced per bud of F_1 progeny from iteroparous and semelparous crosses does not deviate significantly from 1:1 (Grosberg, 1988). The heritability of the iteroparous and semelparous morphs may be related to the single-gene mutations responsible for increased fecundity in two breeds of sheep. Both the X-linked Inverdale gene (Smith *et al.*, 1997) and the Booroola gene (Davis *et al.*, 1982) cause a dose-dependent increase in ovulation rate. The Booroola gene appears to increase ovulation rate by increasing the number of oogonia and primordial follicles in the developing ovary (Smith *et al.*, 1994) and not by elevating hormone levels in the adult (Wheaton *et al.*, 1996). Further work is needed to determine if a homolog of the Booroola or the Inverdale gene is responsible for the iteroparous and semelparous life-history morphs seen in *B. schlosseri*.

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